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Evaluation of an in-house enzyme linked immuonosorbent assay (ELISA) for detection of anti – *Fasciola gigantica* antibodies in sheep

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Abstract

Tropical fasciolosis caused by infection with *Fasciola gigantica* is regarded as one of the most important single helminthes infections of ruminants in Asia and Africa. Fasciolosis diagnosis, due to low sensitivity of coprological diagnostic method has been challenging for a long period. To evaluate the usefulness of ELISA for diagnosis of anti – *Fasciola gigantica* antibodies in parasitized sheep, crude, as well as excretory-secretory (E/S) antigens were examined with 50 positive and 45 negative serum samples. Results were compared with liver inspection in slaughterhouse. The cut off values were determined in 0.469 and 0.516 for crude and E/S antigen respectively. We found 82% of sensitivity, 95.6 of specificity, 95.3% positive predictive value and 83 % negative predictive value using crude antigen. We have also found 92% of sensitivity, 93% specificity, 93.8% positive predictive value and 91.4 % negative predictive value using E/S antigen. We concluded that ELISA, specially using E/S antigen, is a good tool for serodiagnosis of *Fasciola gigantica* infection in sheep herds.

Keywords: *Fasciola gigantica*, ELISA, sheep

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Introduction

Fasciola gigantica in corporation with *Fasciola hepatica* is one of the most economically important parasitic trematode helminthes causing fascioliasis or Liver Fluke in sheep and cattle. These parasites can also infect humans. *F.gigantica* is distributed worldwide and prevalent in most sheep-raising countries (Hillyer, 1986). The parasite settles in the biliary ducts and affects the liver of ruminants and produces significant losses in livestock. Clinical manifestations of this disease in cattle and sheep are anemia and enteritis resulting, eventually in cachexia (Boray, 1997). In Iran the course of the disease is usually referred to as chronic (Rokni *et al.*, 2006).

Usually, stool examination and serological procedures are applied in diagnosis of fascioliasis in ruminants or human populations. Diagnosis of fascioliasis by examination of stool samples lacks sensitivity because the eggs can be found eight weeks post-infection and meanwhile absent in acute and ectopic stages and intermittently released in the chronic stage (Santiago & Hillyer 1988; Makay *et al*., 2007). To resolve this problem, as antibodies of *Fasciola gigantica* infection occurs about two weeks post-infection and because of the simplicity of the assays, and also early seroconversion (usually 1–2 weeks), most investigators today use serological assays for diagnosis of the parasite (Sanchez Andrade *et al*., 2000). Many serological methods have been designed for diagnosing fascioliasis in animal or human such as haemaglutination, indirect fluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (Hillyer *et al*., 1985; Leevieux *et al*., 1992; Rokni *et al.*, 2006). Most of researches have evaluated the serological tests for *Fasciola hepatica* infection, while a few studies have been carried out on *F.gigantica* infection (Fagbemi & Obarisiagbon, 1990).

In the present study we evaluate an ELISA test by crude and excretory-secretory (E/S) antigens to detect the presence of antibodies against *Fasciola gigantica*.

Materials and methods

Sample collection

Blood samples were collected from sheep from Ahvaz slaughterhouse in Khouzestan province. Sheep also were examined macroscopically for the presence of mature and or immature *Fasciola* flukes in their livers, bile ducts and gall bladders according to the method of Anderson *et al.*, (1999). Blood samples were centrifuged in laboratory at 3000rpm for 30 min and sera have kept at −20°C until serologic examination. Totally, 50 sera from parasitized and 50 sera from non-parasitized sheep with *Fasciola* were selected for standardization of the test.

Preparation of crude antigens

Fasciola crude antigens have prepared according to procedure explained by Oldham and Williams (1985) with some modification (Oldham & Williams1985). Adult *F.gigantica* trematods of sheep were briefly washed three times in phosphate buffer saline (PH=7.4), drained and freeze-dried for 24 hours at −70 °C. Then flukes were ground into a fine powder and suspended in PBS and homogenized in a high-speed mixer for15 minutes and allowed to extract overnight at 4 °C. After centrifugation at 3000rpm for 15 min, the supernatant was filtered and sterilized by passing through 0.45µm and 0.22µm filters, then aliquotted and stored at −20 °C until examination.

Preparation of E/S antigens

Fasciola gigantic E/S antigens have prepared according to Simsek *et al*. (2006). To this regard, adult *F. gigantica* trematods of sheep were washed several times in 0.01 M PBS (pH=7.4), then worms were incubated in PBS (5 worms/10 ml) at 37 °C and 5% CO2 for 6 hand removed by sieving and E/S products were centrifuged at 10,000 rpm for

30 min at 4 °C. After that, the supernatant was filtered from 0.22µm filter and products were dialyzed by dialysis tube against distilled water for 24 hours, aliquotted and stored at −20 °C until Examination. Protein concentration of E/S antigens as well as crude antigens was measured according to Lowry *et al.* (1951) method.

ELISA designing

After initial checkerboard titration, optimal antigen, serum and ovine anti-IgG peroxidase conjugate (Sigma) concentrations have determined. Finally, ELISA plates (Nunc, Denmark) were coated with 70µl of crude (6 µg/ml) and 100µl of ES (5.9 µg/ml) antigens solution per well and incubated overnight at ˚4C. The wells were washed three times with PBS (PH=7.4) containing 0.05% Tween and incubated with 150µl of 5% skimmed milk (Merck, Germany) in PBS for an hour at room temperature and then washed three times. After that, 100µl of 1:10 dilution of examined sera were added to each well and incubated for an hour at room temperature. After washing the plate as described, 100µl of 1:7000 of ovine anti-IgG conjugate was added to each well and the plate incubated for an hour at room temperature. As previously described, plate has been washed and then 100µl of substrate (Tetramethylbenzidine + H2O2) was added into each well. After incubation for 15 minutes at room temperature, 100µl of 2N sulfuric acid (Merck, Germany) was added as stop solution.

Finally, the plate was read at 450nm with ELISA reader (Dynatech, Netherland). The cut- off point for the optical density (OD) from the ELISA method was measured with calculating the mean OD of negative sera plus two times of standard deviation (X ± 2 SD). The positive control serum was a pool of sera obtained from slaughtered sheep parasitized with adult liver. Examined sheep were considered accurately for parasitizing with hydatid cyst and *Dicrocoelium dendriticum*.

Results of the ELISA were evaluated in comparison with the results of liver infection.

Results

Cut-off value was calculated by this formula: mean of optical densities of negative sera plus twice mean of standard deviations. The cut-off value for the diagnosing the *F. gigantica* infection in sheep was calculated 0.469 for crude and 0.516 for E/S antigens respectively. Also, after initial designing for performing the tests we adjust the total protein up to 5.9µg/ml and 6µg/ml for crude and E/S antigens respectively. There was no cross reactivity with hydatid cyst and *Dicrocoelium dendriticum*.

The sensitivity, specificity, precision, positive and negative predictive values of ELISA with two mentioned antigens for detecting *F.gigantica* infection in sheep were measured and presented in Table 1.

Table1.Sensitivity, specificity, positive predictive value and negative predictive value percentages of ELISA using Cr and E/S antigens for diagnosis of *F. gigantica* infection in sheep

Antigen type	E/S	Crude
Sensitivity (%)	92	82
Specificity (%)	93	95.6
Precision (%)	92	88.5
Positive predictive value (%)	93.8	95.3
Negative predictive value (%)	91.4	83

Discussion

Fasciolosis due to *F. gigantica* and also *F. hepatica* causes serious disease in ruminants and notable economic loss to the meat industry and also occasionally infects man (Sukapesna *et al*., 1994). Khouzestan is a tropical region and have warm climate that *Lymnea truncatula* (intermediate host of *Fasciola hepatica*) cannot grow and survive in this area, So *Fasciola gigantica* is agent of ovine fascioliasis in this province (Unpublished data).

Serodiagnosis of the *Fasciola* infection is the best option to coprological examinations which reveal the *Fasciola* eggs. The detection of infection by examination of fecal samples for the presence of *Fasciola* eggs is often difficult because they are not found during prepatent period. The advantage of immunodiagnosis is better revealed since detection of *Fasciola* eggs in the feces can be performed eight weeks post infection and clinical signs and symptoms appear three weeks post infection, but antibody appearance occurs about two weeks post infection (Santiago & Hillyer 1988; Anderson *et al.*, 1999).So, if the disease is diagnosed quickly by serological tests, it can be easily treated with specific anthelmintic drugs and we gain even more accurate screening data.

Between all serologic procedures ELISA method is usually sensitive, specific, accurate and easy to perform procedure for paraclinical goals in parasitic infections in endemic areas (Rivera-Morero *et al*., 1988; Hillyer *et al*., 1996; Anderson *et al*., 1999; Kralova-Hramadova *et al*., 2008).

Our study aimed to develop a useful technique for field as well as screening purposes.

In the present study ELISA values of sheep sera parasitized with *F. gigantica* and sheep sera which were free from any *Fasciola* infection in inspection of the liver obtained and we found that with our test designing the titers of parasitized sheep sera in comparison with negative sheep sera using crude and E/S antigens were very different and discriminable. Sensitivity percentages of designed ELISA for diagnosis of ovine fasciolosis using crude antigen was measured as 82% while by using E/S antigen calculated 92%. On the other hand, specificity percentage of ELISA for diagnosis of *F. gigantica* infection in sheep was also measured and high specificity (95.6%) has gained using crude antigen in comparison with using E/S antigen which calculated 93.8%. So, we found the acceptable sensitivity using E/S antigen in comparison with crude antigen while better specificity gained using crude antigen. However, the specificity of the test was also acceptable when we used E/S antigen.

It may be that using different negative sera changes the percentage of these criteria. Vyianat *et al*. (1997) reported that when serum samples from unparasitized calves was used as control cut-off value, the sensitivity was 86.6%, but when the cut-off value was calculated from fetal calf sera and the trematode-free baby calf sera, the sensitivity improved to 100% (Viyanant *et al*., 1997). Anderson et al. reported positive and negative predictive values for ELISA using E/S antigens of *F. gigantica* with 72 sera of stool positive cattle and 20 sera of stool negative cattle were 91.2% and 58.3% respectively (Anderson *et al*., 1999). Also, the sensitivity and specificity of all serologic assays are varying according to the population on which they are tested (Whiting *et al*., 2004; Leeflang & Bossuyt 2005) and the season at which samples are being tested (Charlier *et al*., 2008).

Crude antigens has numerous variety of antigenic components compared to those of E/S antigens which may lead to more sensitivity and less specificity using E/S antigens. Furthermore, no test currently available is considered to have both 100% sensitivity and 100% specificity.

False positive cases detected by ELISA may be attributed to recent administration of effective fasciolicides which eliminated

adult flukes from the biliary ducts but specific antibodies still persist in serum for 2–7 months (Santiago & Hillyer 1988; Levieux *et al*., 1992; Castro *et al*., 2000). Also this may be due to the possession of common antigens by different helminthes (Fagbemi & Obarisiagbon 1991).On the other hand it is possible that the sampled sheep were previously exposed to *F.gigantica* infection and antibody to the infection already existed.

The false negative results may be attributed to modulation of the host immune response by liver flukes as reported by (Zimmerman *et al*., 1983) who recorded a significant immune suppression during *F. hepatica* infection.

In conclusion, the present study demonstrates that our ELISA for the detection of *Fasciola gigantica* is able to detect early with acceptable sensitivity and specificity specially, when we use E/S proteins of trematode as antigen. However, this method has limitations because the presence antibodies indicate previous exposure to the parasites rather than the existence of a current infection. So, further investigation should be performed by a detection of immunoglobulin subclass or by preparation of antigen with different molecular weight cut-off technique.

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ارزیابی آزمایش الایزا در تشخیص آنتی بادی ضد فاسیولا ژیگانتیکا در گوسفند

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چکیده

فاسیولوز مناطق گرمسیری عمدتاً به دلیل آلودگی نشخوارکنندگان به انگل فاسیولاژیگانتیکا در آسیا و آفریقا دیده می‌شود. تشخیص فاسیولوز به دلیل حساسیت کم آزمایش مدفوع یکی از مسائل چالش برانگیز در این بیماری می باشد. به منظور ارزیابی آزمایش الایزا در تشخیص فاسیولوز گوسفند با استفاده از آنتیژن های خام و دفعی- ترشحی انگل تعداد ۵۰ نمونه سرم مثبت و ۴۵ نمونه سرم منفی مورد سنجش قرار گرفته و نتایج با بازرسی پس از کشتار مقایسه گردیدند. ارزش cut-off برای آنتی ژن خام ۰/۴۶۹ و برای آنتی ژن دفعی- ترشحی ۰/۵۱۶ تعیین گردید. با استفاده از آنتی ژن خام حساسیت، ویژگی، ارزش اخباری مثبت و ارزش اخباری منفی به ترتیب ۸۲ درصد، ۹۵/۶ درصد، ۹۵/۳ درصد و ۸۳ درصد و با استفاده از آنتی ژن دفعی- ترشحی مقادیر مذکور به ترتیب ۹۲ درصد، ۹۳ درصد، ۹۳/۸ درصد و ۹۱/۴ درصد محاسبه گردیدند. با توجه به نتایج این مطالعه انجام آزمایش الایزا با استفاده از آنتی ژن دفعی- ترشحی می تواند یکی از ابزارهای تشخیص فاسیولوز گوسفند در مناطق گرمسیری باشد.

واژگان کلیدی: فاسیولا ژیگانتیکا، الایزا، گوسفند